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Short communication

A remarkable new species of *Geastrum* with an elongated branched stipe

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ABSTRACT

Based on morphological and molecular analysis, we describe the new species *Geastrum verrucoramulosum*, discriminated from other species in the section *Exareolata* mainly by an elongated, verrucose, branched stipe. This new species is currently known from two forest locations in central and southwestern Amazonia. Species description, images, and taxonomic discussion of both morphological and molecular data are provided.

The order Geastrales K. Hosaka & Castellano (Hosaka et al. 2006) has been the subject of studies to elucidate the phylogenetic relationships among its genera and species (Hosaka and Castellano 2008; Kasuya et al. 2012; Zamora et al. 2013, 2014). However, the diversity of tropical earthstars is still poorly known. New discoveries and the inclusion of DNA sequences of tropical species are needed, and may modify the current phylogeny.

In recent years, intensive fieldwork focusing on gasteroid fungi (Basidiomycota) has been carried out in several Brazilian biomes, including the Semi-arid, Atlantic Rainforest, and Amazon Rainforest, especially focusing on *Geastrum* species

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(Sousa et al. 2014a, b; Cabral et al. 2014b). These studies have revealed exceptional new and unusual diversity, totaling 54 species described so far for Brazil (Baseia and Milanez 2002; Baseia and Calonge 2006; Fazolino et al. 2008; da Silva et al. 2013; Cabral et al. 2014a; Sousa et al. 2015). In this study, our goal is to provide new information about the genus *Geastrum* in the Amazon Rainforest by describing a remarkable new species with an elongated and branched stipe.

Specimens were collected during the rainy season of 2012 in the Brazilian Amazon rainforest. One specimen (LABEV 6059) was collected during an ecology field course conducted by the Federal University of Acre (UFAC) and the Research Program on Biodiversity (PPBio), and sent to the first author by the collectors. Descriptive terminology and taxonomy are based on Sunhede (1989), Baseia and Milanez (2002), and da Silva et al. (2013). Color codes follow Kornerup and Wanscher (1978). For light microscopy, free-hand sections were mounted in 5% (w/v) KOH and then examined with a Nikon Eclipse Ni light microscope (Nikon Corporation, Tokyo) coupled with a Nikon DS-Ri camera (Nikon Corporation, Tokyo), supported by NIS-Elements AR 4.00.03 software (Nikon Corporation, Tokyo). Thirty randomly selected basidiospores were measured using light microscopy, under the 100× immersion oil objective, with 10× oculars. All measurements include basidiospore ornamentation. Basidiospore abbreviations follow Sousa et al. (2014a): n = number of randomly measured basidiospores; x = mean ± standard deviation of basidiospore diameter and height (including ornamentation); Q_m = mean height/width quotient. Scanning electron microscopy studies were performed at the Universidade Federal do Rio Grande do Norte (UFRN) with a Philips XL 20 (Philips Company, Amsterdam), in accordance with previously described methods (da Silva et al. 2011). Specimens are deposited in the fungal collection of the INPA and UFRN Herbaria (Manaus and Natal, Brazil).

DNA extraction was performed from a small piece of the dried basidioma, following da Silva et al. (2013). Two DNA regions were amplified, the nuclear large subunit rDNA (nuc-LSU) and the mitochondrial ATPase subunit 6 coding region (*atp6*), using primers developed by Vilgalys and Hester (1990) and Kretzer and Bruns (1999). The PCR fragments were purified with ExoSAP-IT (Affymetrix Inc., Thermo Fisher Scientific, Waltham), and sequenced with BigDye™ Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (Applied Biosystems™, Thermo Fisher Scientific, Waltham). The nuc-LSU and *atp6* sequences generated in this study, and those published by Zamora et al. (2014) and Cabral et al. (2014b), were aligned and manually edited in Geneious R6.1 (Biomatters Ltd., New Zealand), treating each DNA region separately. Two datasets were analyzed. The first was used to determine in which section the new species belongs; preliminary analyses were done including sequences of representatives from all sections (alignment 1), according to Zamora et al. (2014), and using *Myriostoma coliforme* (Dicks.) Corda as outgroup. In order to delimitate the new species, the second dataset was analyzed using only representatives of the section in which the new sequences belong, and using *G. schmidelii* Vittad. as outgroup (alignment 2) (Table 1).

The GTR substitution model was chosen by MrModelTest 2.3 (Nylander 2004) for both *atp6* and nuc-LSU. The two blocks of aligned sequences from each DNA region were concatenated to form one single matrix. This matrix was used to perform maximum parsimony and Bayesian phylogenetic analyses. The maximum parsimony analysis was conducted with PAUP* 4.0 (Swofford 2003). The trees were calculated with a heuristic search, with branch swapping, using the TBR algorithm, with initial trees obtained by stepwise addition of random additional sequences repeated 100 times, and bootstraps of 1000 replicates. The Bayesian analysis was performed with MrBayes v.3.1.2. (Huelsenbeck and Ronquist 2001), where trees were calculated using two different runs with four incrementally heated simultaneous MCMC simulations over 10 million generations for the first analysis and 2 million for the second analysis, with trees sampled at every 1000 generations. To estimate posterior probabilities and calculate the consensus tree, part of the trees was discarded as a burn-in stage observing the average standard deviation of split frequency values. The trees were edited with FigTree (http://tree.bio.ed.ac.uk/software/figtree/). All molecular data can be accessed at TreeBase under ID 19668.

Alignment 1 consisted of 133 taxa and 1512 characters (862 nuc-LSU and 649 *atp6*), among which 508 were parsimony-informative characters. The maximum parsimony and Bayesian analyses resulted in similar phylogenetic trees, where the new species clustered with species from section *Exareolata* De Toni (trees not shown; see TreeBase ID 19668). In alignment 2, only species from section *Exareolata* were included, with *G. schmidelii* as outgroup. The concatenated matrix consisted of 16 taxa and 1496 characters (906 corresponding to nuc-LSU and 589 corresponding to *atp6*), among which 236 were parsimony-informative characters. We obtained one most parsimonious tree with 543 steps and CI = 0.705, RI = 0.788, RC = 0.555. Both maximum parsimony and Bayesian analyses resulted in similar trees (Fig. 1), where the new species is a sister clade of *Geastrum* cf. *stipitatum* [determined in Zamora et al. (2014)], also from Brazilian Amazon Rainforest. Due to the unique LSU and *atp6* sequences, as well as unique morphological characters, the new species *Geastrum verrucoramulosum* is here described.

Taxonomy

Geastrum verrucoramulosum T.S. Cabral, J.O. Sousa, & Baseia, sp. nov. Figs. 2–4.
MycoBank no.: MB817844.

Diagnosis: Unexpanded basidioma epigeous, caespitose, surface densely verrucose, developed above a prominent and ramulose stipe (17–41 mm high). Endoperidium comprised of irregularly arranged hyphae. Peristome not truly plicate, becoming fibrillose with age, not delimited. Columella circular. Basidiospores globose, 3.6–4.5 × 3.6–4.4 μm, verrucose, short warts, columnar, with flattened to rounded apex.

Type: BRAZIL, Amazonas, Manaus, Estação Experimental de Manejo Florestal ZF-2, on clay soil, 17 Mar 2012, leg. D.L. Komura 286 (holotype, INPA 264956), Genbank KX670829 (*atp6*), KX670831 (nuc-LSU).

Etymology: *verrucoramulosum* (Lat.), referring to the ramulose stipe and the verrucose surface of the exoperidium.

Table 1 – Sequences used in alignment 2. Species names, herbarium vouchers, localities, and Genbank accession numbers.

Species	Herbarium voucher	Locality	nuc-LSU	*atp6*
Geastrum albonigrum	UFRN-Fungos 1989	Brazil	KJ127019	KJ127015
Geastrum albonigrum	MA-Fungi 36140-2	Panama	KF988468	KF988738
Geastrum aculeatum	UFRN-Fungos 1681	Brazil	JQ683661	JQ683668
Geastrum argentinum	LSP 48446	Argentina	KF988472	KF988742
Geastrum argentinum	MA-Fungi 82605	Argentina	KF988473	KF988743
Geastrum echinulatum	INPA 240001	Brazil	JQ683659	JQ683665
Geastrum echinulatum	INPA 240005	Brazil	JQ683660	JQ683666
Geastrum inpaense	INPA 239990	Brazil	KJ127017	KJ127013
Geastrum inpaense	INPA 255834	Brazil	KJ127018	KJ127014
Geastrum rufescens	Zamora 274	Spain	KF988553	KF988820
Geastrum rufescens	Zamora 253	Spain	KF988552	KF988819
Geastrum schmidelii	UPS F-560805	Sweden	KF988565	KF988832
Geastrum schmidelii	Zamora 279	Spain	KF988564	KF988831
Geastrum cf. *stipitatum*	Zamora 528	Brazil	KF988576	–
Geastrum verrucoramulosum	INPA 264956	Brazil	KX670831	KX670829
Geastrum verrucoramulosum	LABEV 6059	Brazil	KX670832	KX670830

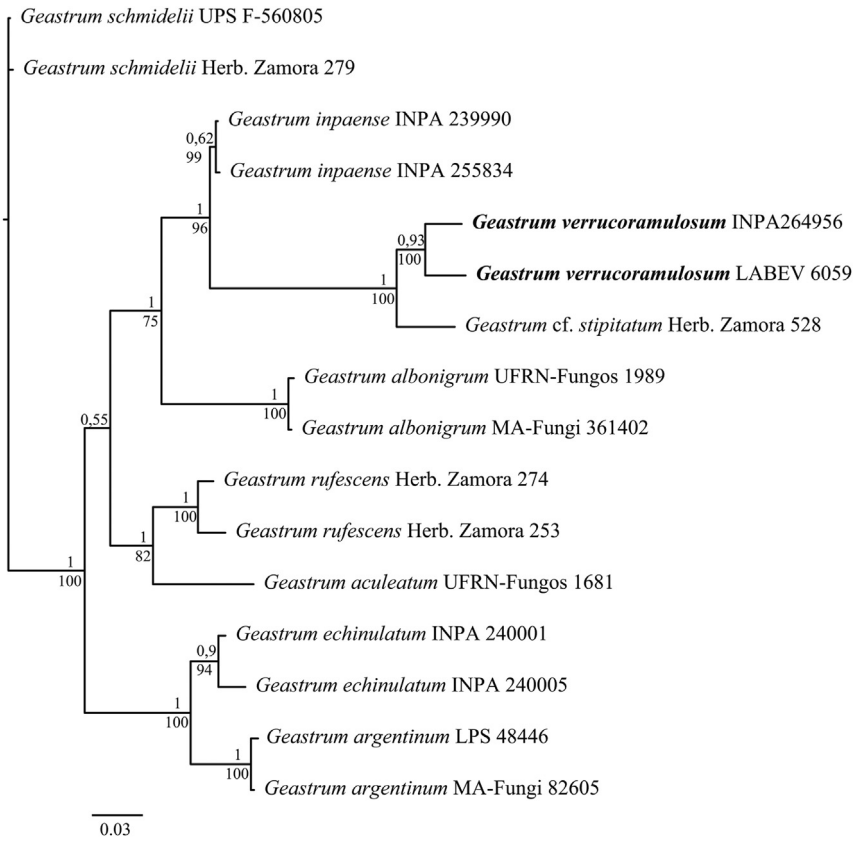

Fig. 1 – Phylogenetic tree obtained by Bayesian analysis derived from concatenated data (*atp6* and nuc-LSU), with representatives of section *Exareolata*. Codes after species names are herbarium vouchers; in bold the new species *Geastrum verrucoramulosum*. Numbers on nodes indicate support values (posterior probabilities values above, and percentage of bootstrap below) and the scale bar indicates substitution per site.

Unexpanded basidiomata epigeous, caespitose, subglobose, 9–17 mm high (not including stipe) × 6–13 mm wide, surface densely verrucose (warts pyramidal, up to 1.5 mm high), rugose with age, light brown (6D4) to brown (6E4), not encrusted with debris, developed above a prominent stipe, absence of a subiculum. Stipe ramulose, trumpet-like, 17–41 mm high × 4–6 mm wide, surface longitudinal-striated, yellowish brown (5D5) to yellowish (5D4). Expanded basidiomata saccate, 8–15 mm high (not including stipe) × 15–19 mm wide. Exoperidium splitting into 6–7 triangular rays; rays triangular, often involute, non-hygroscopic. Mycelial layer dark brown (7F3), surface rugose, not encrusted with debris, persistent. Fibrous layer yellowish white (4A2), papery. Pseudoparenchymatous layer light brown

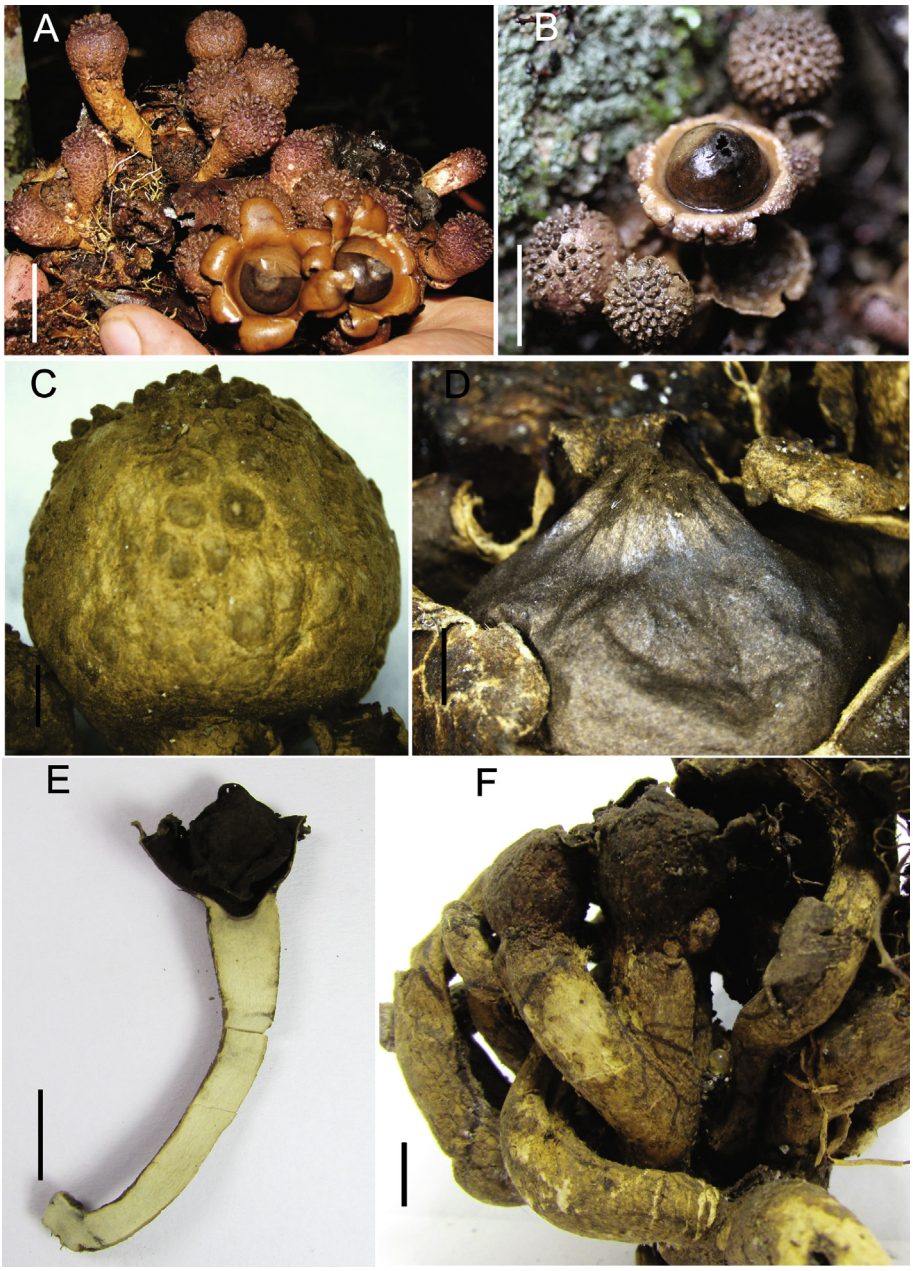

Fig. 2 – *Geastrum verrucoramulosum* sp. nov., fresh (A, B) and dried (C–F) basidiomata. A: LABEV 6059, paratype (Photo: Wendeson Castro). B: INPA 264956, holotype (Photo: D.L. Komura). C–F: UFRN-Fungos 2782, paratype (Photos: Wendeson Castro). C: Exoperidium with densely verrucose surface. D: Non-delimitated peristome. E: Cross-section expanded basidioma. F: Ramulose stipe. *Bars*: A, B 10 mm; C–E 2 mm; F 5 mm.

(6D8) when fresh, becoming brownish gray (8F2) when dry, peeling away in irregular patches from the base of the rays, persistent. Endoperidium subglobose, 9–10 mm high × 8–10 mm wide, sessile, apophysis absent, surface glabrous, grayish brown (7F3). Peristome not truly plicate, becoming fibrillose with age, non-delimitated, conic (up to 3 mm high), lighter than the endoperidium. Columella circular, central, 1.5 mm wide, yellowish white (4A1) in cross-section. Gleba pulverulent, grayish brown (8F3).

Warts from outer part of peridium composed of thin-walled (<1 μm) sphaerocysts, subglobose, pyriform to oval, 14.4–30.7 × 13–27.4 μm, brownish. Mycelial layer composed of thin sinuous-walled (<1 μm) hyphae, 3.8 × 5.7 μm diam, surface not encrusted, lumen evident, hyaline to yellowish. Fibrous layer composed of thin-walled hyphae (<1 μm), 3.7–6.3 μm diam, surface not encrusted, lumen evident, hyaline. Pseudoparenchymatous layer composed of thick-walled (>1 μm) hyphal cells, subglobose, oval to pyriform hyphae, 24–52.4 × 21–32.9 μm, brownish. Endoperidium comprised of irregularly arranged hyphae, 2.4–2.6 μm diam. Stipe composed of filamentous, thick walled hyphae (0.5–1.0 μm), 4.7–5.9 μm diam, surface encrusted with amorph material, lumen evident, light brown to yellowish. Eucapillitium 3.8–6.2 μm diam, thin walls (<1 μm), surface rugulose,

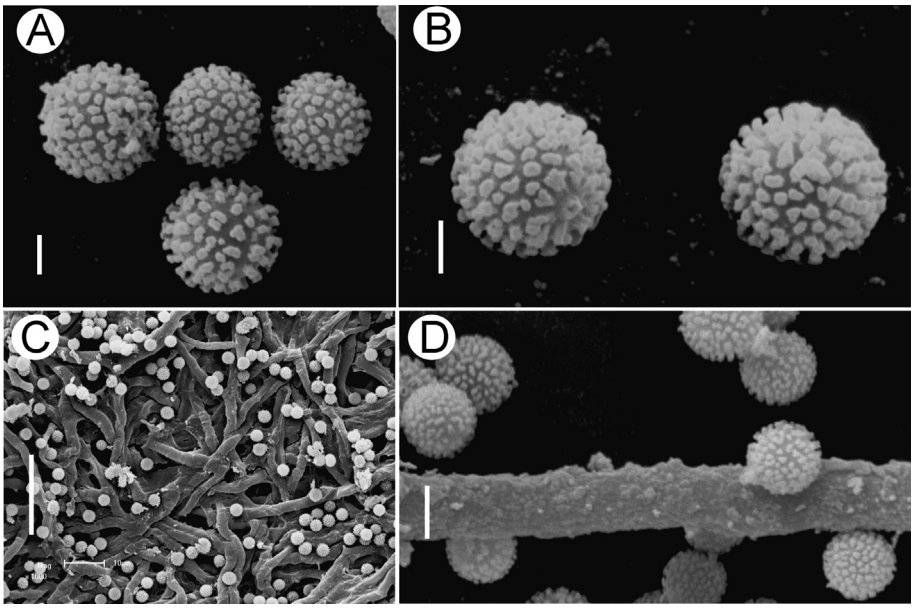

Fig. 3 – *Geastrum verrucoramulosum* sp. nov., micro-structures of UFRN-Fungos 2782 (paratype) under scanning electron microscope (Photo: Iuri G. Baseia). A, B: Basidiospores. C: Hyphae of endoperidium surface. D: Hyphae of eucapillitium. *Bars*: A, B 1 μm; C 20 μm; D 2 μm.

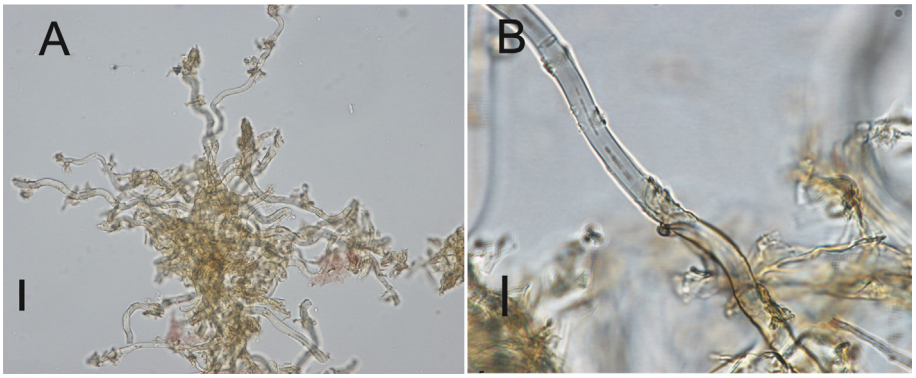

Fig. 4 – *Geastrum verrucoramulosum* sp. nov., micro-structures of stipe (A, B). A: Under 400× of light microscope. B: Under 1000× of light microscope. *Bars*: A 20 μm; B 10 μm.

strongly encrusted, lumen evident, branched, yellowish brown. Basidia 7.3–32 × 2.8–7.5 μm, clavate, guttulate, 2 sterigmata. Basidiospores globose, 3.6–4.5 × 3.6–4.4 μm [$x = 4.0 \pm 0.2 \times 4.0 \pm 0.2$, $Q_m = 1.01$, $n = 30$], strongly brownish, warts inconspicuous under light microscope, verrucose under SEM; warts short, columnar, with flattened to rounded apex; apiculus inconspicuous.

Habitat and distribution: terrestrial, found both in open forest composed mainly of shrubs on white-sand soil (called 'campinarana') and in upland dense forest on clay soil. So far the new species is restricted to the Amazon Rainforest.

Additional specimen examined (paratype): BRAZIL, Acre, Mâncio Lima, Santa Bárbara community (Módulo do PPBio, LO I parcela P50), on white-sand soil, 30 Nov 2012, leg. J.C.N. Rosario 25 (LABEV 6059, UFRN-Fungos 2782), Genbank KX670830 (*atp6*), KX670832 (nuc-LSU).

The molecular phylogenetic analyses placed the new specimens from Amazon Rainforest within the genus *Geastrum*. Based on the phylogenetic tree and morphological analyses, it can be confirmed that the new species belongs to the section *Exareolata*. Despite the morphological heterogeneity of this section, *G. verrucoramulosum* shares characteristics with other species assigned to this section, such as: exoperidium with well-developed stipe, non-delimited peristome, and mycelial layer subdivided into two layers (Zamora et al. 2014). This species is grouped in a clade with other species included by Zamora et al. (2014) in the *Exareolata* section, such as *Geastrum aculeatum* B.D.B. Silva & Baseia, and *G. echinulatum* T.S. Cabral, B.D.B. Silva & Baseia. These species present exoperidium with protruding hypha, non-delimited peristome and saccate basidiomata like *G. verrucoramulosum*, but they are easily distinguished by the absence of a stipe and the morphology of exoperidium tufts, which in *G. verrucoramulosum* are warts composed of sphaerocysts.

Geastrum verrucoramulosum presents a prominent branched stipe below the basidiomata, making it unique.

The presence of the long branched stipe can be considered an autapomorphy within the genus, found only in the new species described here. Furthermore, this species has a range of characteristics that together makes it distinct from the rest of the genus: caespitose growth, epigeal development, non-delimited peristome, saccate basidiomata, sessile endoperidium, verrucose exoperidium and spores with short warts.

One species that presents a stipe below the basidiomata is *Geastrum stiptatum*, described by Solms as '*Geaster stipitatus*' (Fischer 1893), distributed throughout Java, the Congo, and Brazil (Lloyd 1907; Dissing and Lange 1962). Another species that also presents a stipe is *Geastrum congolense* Dissing and Lange (1962). Mainly because stipe development started from a subiculum, Ponce de León (1968) proposed the combination of *G. stipitatum* and *Geastrum congolense* in *Geastrum schweinitzii* var. *stipitatum* (Solms) P. Ponce de León. *Geastrum schweinitzii* var. *stipitatum* presents characteristics in common with *G. verrucoramulosum*, such as saccate basidiomata, caespitose habit and the presence of pyramidal warts in the external portion of the peridium. However, *G. schweinitzii* var. *stipitatum* is different from *G. verrucoramulosum* by having a less conspicuous unbranched stipe (up to 10 mm in height × up to 3 mm in width), the presence of a subiculum, delimited peristome, and smaller spores (up to 3.5 μm in diam) that are sparsely ornamented (Dissing and Lange 1962; Ponce de León, 1968; Calonge and Daniëls 1998). Another species described as having a stipitate exoperidium is *Geastrum juruense* Henn. (as '*juruensis*') described from Amazon Rainforest in 1904, and it is different from *G. verrucoramulosum* by having a smaller unbranched stipe (up to 10 mm in height), and presence of subiculum and smaller basidiospores (up to 2.5 μm in diam) (Hennings 1904).

The caespitose form of development of the basidiomata is a habit observed in *G. verrucoramulosum* and shared by a restricted group of earthstar species that also occur in tropical regions, such as *G. schweinitzii* (Berk. & M.A. Curtis) Zeller, *G. schweinitzii* var. *stipitatum* and *G. hirsutum* Baseia & Calonge. These species are differentiated from *G. verrucoramulosum* by the presence of subiculum, delimited fibrillose peristome, mycelial layer with prominent hyphae (hirsute or tomentose), and absence of a prominent unbranched stipe under the basidiomata (Dissing and Lange 1962; Ponce de León 1968; Calonge and Daniëls 1998; Baseia and Calonge 2006).

The basidiospores found in *G. verrucoramulosum* present short warts with flattened or rounded apex, inconspicuous under light microscope, very similar to the warts found on the basidiospores of *G. hirsutum*. Nevertheless, these species are easily separated, for *G. hirsutum* presents a hairy mycelial layer, delimited peristome, presence of a subiculum, and the absence of a branched stipe under the basidiomata (Baseia and Calonge 2006).

Disclosure

The authors declare no conflicts of interest. All work undertaken in this study complies with the current laws of the country where they were performed.

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